UNIVERSITY OF ALBERTA

CHARACTERIZATION OF THE MECHANISM OF ANF-INDUCED FLUID EXTRAVASATION FROM THE SPLEEN

BY

RICHARD A. SULTANIAN ©

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ABSTRACT

This study was undertaken to determine the mechanism underlying ANF-mediated increases in fluid extravasation from the spleen. Experiments were done in an isolated, blood-perfused rat spleen. The double occlusion technique was used to determine alterations in splenic hemodynamics in response to ANF. ANF dose-dependently increased both intrasplenic capillary pressure and post-capillary resistance. ANF also increased the splenic arterio-venous flow differential, a measure of fluid extravasation.

The NPR_C specific agonist C-ANF did not cause any significant alterations in splenic hemodynamics. However, the addition of A71915, an ANF-antagonist, completely blocked the actions of ANF in the spleen. Since A71915 blocks the production of cGMP by ANF, it is concluded that ANF mediates its hemodynamic actions via the activation of NPR_A. Therefore, ANF, which is released in response to hypervolemia, is able to significantly decrease intravascular blood volume through hemodynamic alterations in splenic capillary filtration pressure, which significantly increases splenic fluid extravasation.

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHARACTERIZATION OF THE MECHANISM OF ANF-INDUCED FLUID EXTRAVASATION FROM THE SPLEEN, submitted by RICHARD A. SULTANIAN in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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LIST OF ABBREVIATIONS

ANF	Atrial Natriuretic Factor
cGMP	cyclic Guanosine Monophosphate
ET	Endothelin
LPS	Lipopolysaccharide
MAP	Mean Arterial Pressure
MAPK	Mitogen-activated Protein Kinase
NO	Nitric Oxide
NPR	Natriuretic Peptide Receptor
P_C	Capillary Pressure
PKG	Protein Kinase G
R_A	Pre-capillary Resistance
R_V	Post-capillary Resistance

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

A. The Spleen:

i). *History*

Many attempts have been made throughout history to understand the structure and function of the spleen. Galen (121-201 AD) called the spleen an "organ of mystery", which, for the most part, still holds true (28). The first insight into the relationship between the circulating blood and the spleen was made by van Leeuwenhoeck (1632 – 1723 AD), who suggested that the spleen plays a role in purification of the blood. This idea was supported in 1854 by Gray, whose *Textbook of Anatomy* described that the function of the spleen is to 'regulate the quantity and quality of the blood' (19). This precipitated a great deal of research into the control of the quantity of circulating red blood cells by the spleen, which is unfortunate considering that the physiological storage of blood cells is not a significant feature of the human spleen (19); with a red cell content of only 30-50 ml, the human spleen has almost negligible blood storage capabilities (30, 46).

ii). *Structure and Function*

Significant progress has been made in the last 50 years in regards to understanding the anatomy, physiology and pathophysiology of the spleen. The human spleen is about the size and shape of a clenched fist (4). It is a fragile

vascular lymphatic organ that is found in the abdominal cavity lying superior and posterior to the cardiac end of the stomach at the level of the 9th, 10 and 11th ribs, with its long axis parallel to them (28). The human spleen weighs approximately 150 g in adults (46). Resting splenic blood flow in all species has been reported to lie between 40-100 ml min^{-1} per 100 g of tissue (87). This is significant as it corresponds to 1-10% of total cardiac output.

The spleen is the largest aggregation of lymphoid tissue, and it is the only lymphoid organ specialized for the filtration of blood (102). The spleen is dedicated to the clearance of defective or old blood cells, microorganisms and other particles from the blood. In humans and rats, the spleen is involved in the primary immune response to blood-borne antigens; it also acts as a regulator of immune reactions occurring elsewhere in the body (24).

The rat spleen is enclosed by a capsule composed of dense white connective tissue that contains very little vascular smooth muscle (87). While the human spleen is slightly more contractile, its ability to contract is insignificant when compared to animals like the horse or dog (87). The inner surface of the capsule gives rise to the splenic trabeculae and pulp, which subdivides the spleen into a series of communicating compartments. The splenic pulp is composed of the white pulp, the marginal zone and the red pulp (97).

The white pulp consists predominantly of lymphocytes, macrophages and other free cells, lying in a specialized reticular meshwork of stromal cells. The white pulp acts as the splenic filtration bed which selectively filters and sorts lymphocytes and accessory cells, setting them up to carry out immune responses throughout the body (24). The marginal zone links the white pulp to either the red pulp or directly into the venous sinuses. Over 75 % of the volume of the spleen is made up of the red pulp, which is composed of reticular tissue rich in capillary beds and penetrated by venous sinuses (44, 45). The red pulp is the splenic filtration bed that is responsible for the majority of fluid extravasation from the plasma into the lymphatic system. The spleen is characterized by an open circulation, in which there is no endothelial continuity between the terminal arterioles and the proximal veins (4). This makes the spleen freely permeable to plasma proteins, which has been demonstrated by the iso-oncotic nature of lymph fluid draining from the splenic vasculature (58).

iii). ***Splenic Blood Flow***

The primary source of blood into the spleen is the splenic artery, which subdivides into the superior and inferior branches before entering the spleen at the hilus (4). The afferent blood and lymph vessels are then carried into the splenic pulp within the trabeculae (Figure 1). There are two different routes of blood flow through the spleen. The first route is called the 'fast pathway' and consists of blood flowing from the trabecular arteries, through the white pulp and marginal zone and then directly into the trabecular veins, with only a small percentage

flowing through the red pulp and venous sinuses (45). The existence of arterio-venous shunts has been demonstrated in the spleen, and blood flowing through these shunts completely bypass the filtration sites within the spleen (45). While the 'slow pathway' follows a similar route through the spleen, a much larger percentage of the total splenic blood flows through the red pulp, where plasma fluid is subsequently extravasated into the systemic lymphatic system (71, 111). However, the blood flow pathway through the spleen depends of the physiological state of the body. Under normovolemic conditions, 90 - 93% of the arterial blood flows through the spleen via the fast pathway, with an erythrocyte washout time of 30 seconds (46). The remaining 7 - 10% of the blood follows the 'slow pathway' through the reticular meshwork of the red pulp for filtration in 8 – 54 minutes (46). Under conditions of hypervolemia, the majority of splenic blood flows through the slow pathway in order to increase intrasplenic filtration of plasma volume (45). In contrast, during conditions of hypovolemia, such as hemorrhage, the total blood flowing through the fast pathway is increased from 90 to 98.7%, with an erythrocyte washout time of 20-30 seconds (45, 66).

iv). *Splenic Control of Blood Volume*

Due to the considerable interspecies variability in the structure and function of the spleen, investigating splenic physiology still presents a major challenge to modern researchers. The rat was chosen as our experimental animal model to investigate the ability of the spleen to control fluid volume homeostasis. The benefit of using this model is that the rat spleen is very similar to the human spleen, in that it is

non-contractile and has virtually no blood storage capacity (87). Previous experiments from our lab have shown that splenic venous hematocrit is consistently higher than splenic arterial hematocrit under normal physiological conditions (58). Further research demonstrated that there is increased intrasplenic filtration of plasma out of the blood and into the systemic lymphatic system in response to hypervolemia, atrial distention and ANF (26).

B. Atrial Natriuretic Factor

Hypervolemia increases atrial pressure, cardiac output and mean arterial pressure (17). These changes stimulate the cardiopulmonary and arterial baroreceptors, which initiate movement of fluid and electrolytes out of the intravascular compartment and an increase in renal output. By these mechanisms the body achieves both the redistribution and elimination of excess intravascular volume and extracellular fluid (20). Atrial natriuretic factor (ANF) is an important peptide that acts to maintain fluid volume homeostasis. It is primarily released in response to atrial distention, as would normally occur during hypervolemia (68).

i). ***Background***

In 1981 de Bold et al published the first study showing that the infusion of atrial tissue extracts into rats caused copious natriuresis (31). This led to the isolation and cloning of atrial natriuretic factor, the first member of a family of natriuretic peptides with diverse biological activities regulating renal and cardiovascular homeostasis. The natriuretic peptide family consists of three peptides: atrial

natriuretic factor (ANF), brain natriuretic peptide (BNP) and c-type natriuretic peptide (CNP). Although structurally similar, these peptides are encoded by separate genes and differ in their tissue distribution, receptor affinities and biological actions (79).

ii). ***Molecular Biology***

ANF is produced primarily in the cardiac atria, where it is initially synthesized as a preprohormone 152 amino acids in length. The principal form of ANF storied in atrial granules is the 126 amino acid prohormone, which is formed after the proteolytic cleavage of the preprohormone. The highest concentrations of atrial storage granules are found in the atrial appendage, which is the primary site where ANF is released into the circulation. The active form of ANF released from the atrial granules is a 28 amino acid peptide that has a disulfide bridge between two of its cysteine residues; all ANF analogues with natriuretic or diuretic activity share this common central ring structure (61). Very little ANF is produced by ventricular tissue in normal adults, but it is present in the ventricular tissue of fetuses and neonates as well as in hypertrophied ventricles (89, 112).

iii). *ANF Release*

The mechanism controlling the release of ANF remains uncertain despite extensive investigation. ANF is released in response to increases in atrial wall distention and/or tension, such as occurs during intravascular volume expansion (i.e. hypervolemia) (57). However it is unknown if it is the stretch itself that results in ANF secretion, or if the stretch causes the release of a second messenger that subsequently causes the release of ANF. In isolated hearts or atria, atrial stretch causes only a short-lived ANF secretion lasting a few minutes (54). Thus, atrial stretch does not fully account for the sustained increase in plasma ANF concentration commonly observed in hypervolemia.

Stimulation of the sympathetic nervous system is also able to increase the release of ANF from the heart (88). Norepinephrine, as well as other more selective α- and β-adrenergic agonists, has been shown to stimulate ANF release *in vitro* and *in vivo* (29, 86, 91). However, one must be cautious that the observed effects of sympathomimetics on ANF release are not secondary to changes in systemic hemodynamic variables. While it is most likely that the increase in ANF by α- and β-adrenergic agonists is indirectly mediated via increased atrial stretch or pressure (118), a direct stimulatory effect on ANF release was revealed when increases in heart rate, myocardial contractility and total peripheral resistance were minimized (14, 60).

Recent studies have shown that several hormones and neurotransmitters are capable of modulating the release of ANF from the heart (34). Vasoconstrictors such as epinephrine, arginine vasopressin and angiotensin II all cause ANF release (22, 23, 90). In particular, there is a considerable amount of data supporting a role for endothelin in mediating ANF release. Endothelin has potent ANF releasing activity in all types of *in vivo* and *in vitro* preparations, and it is a primary candidate as a paracrine or endocrine regulator of stretch-induced ANF release (13, 88).

iv). *Receptor Physiology*

Molecular cloning and radio-ligand binding studies have identified three different natriuretic peptide receptors (NPR). Two of these, NPR_A and NPR_B, are linked to membrane bound guanylyl cyclase (Figure 2) (98). These receptors are single transmembrane proteins, consisting of an extracellular domain that confers natriuretic peptide binding specificity, and an intracellular region consisting of a guanylyl cyclase domain, which is activated upon binding of the appropriate natriuretic peptide to the extracellular domain (7). Activation of NPR_A and NPR_B has been linked to cGMP and the cGMP-dependent signaling cascade in a variety of *in vivo* and *in vitro* studies, and is thought to mediate many of the cardiovascular and renal effects of natriuretic peptides (36, 69). In vascular smooth muscle, the cGMP production by ANF stimulates a cGMP-dependent

protein kinase (PKG), which acts at different locations to attenuate intracellular Ca^{2+} levels (76, 78).

The third member of the NPR family is NPR_C. While the extracellular domain of NPR_C is homologous to the NPR_A and NPR_B, the intracellular domain consists of a very short cytoplasmic tail that lacks any guanylyl cyclase activity (6). The NPR_C-subtype was initially believed to function only as a clearance receptor (70). Once the natriuretic peptides bind to NPR_C, they are internalized and then enzymatically degraded, after which the receptor returns to the cell surface. However, recent evidence has demonstrated that NPR_C activation is also associated with the inhibition of both adenylyl cyclase and mitogen-activated protein kinase activity (MAPK) (6, 52). The NPR_C-subtype has been shown to represent over 90% of the total ANF receptor population in most tissues and vascular beds (7).

The three NPR-subtypes have different binding specificities for the natriuretic peptide family. Both ANF and BNP preferentially bind to NPR_A, even though BNP is approximately 10 times less potent than ANF in stimulating cGMP production. In contrast, CNP has very low affinity for NPR_A, but is the only natriuretic peptide that binds to NPR_B (61). All three natriuretic hormones bind to NPR_C with equal affinity.

v). ***ANF Metabolism***

Atrial natriuretic factor is rapidly cleared from the circulation. In man and other animals half-lives of between 1 and 4 minutes have been reported (3, 103, 114). While the process of clearance from the cardiovascular system is still not full understood, at least two mechanisms are thought to be involved. First, ANF is metabolically degraded by a zinc-dependent neutral endopeptidase (NEP) found on vascular smooth muscle and endothelial cells, and secondly, ANF is removed from the circulation through binding to NPR_C (82). Studies using NEP inhibitors such as candoxatrilat, and the blockade of ANF clearance receptors with C-ANF (a NPR_C-specific agonist) have shown that both of these mechanisms play major and almost equal roles in the rapid clearance of ANF from the circulation (15, 25).

vi). ***Effects of ANF on the Cardiovascular System***

Cardiovascular effects of ANF include hypotension, fluid leakage from the vasculature, vasodilation, increased venous capacitance and inhibition of mitogenesis (5). Even though vasodilation is a commonly accepted physiological action of ANF, the hypotension caused by ANF has often been attributed to a decrease in cardiac output rather than to a fall in total peripheral resistance (115). In fact, vasodilation is not consistently observed in response to ANF infusions *in vivo*, unless large bolus doses are used (115). The decrease in blood pressure caused by ANF is also due to a reduction in cardiac preload caused by fluid extravasation from the intravascular compartment into the extravascular compartment (33). While the mechanism behind this activity is still unclear, this

could reflect an increase in capillary permeability or an increase in capillary hydrostatic pressure in different vascular beds of the body.

Atrial natriuretic factor is capable of reducing sympathetic tone in the peripheral vasculature. This reduction is caused in a variety of ways, such as dampening of baroreceptors, suppressing catecholamine release from autonomic nerve endings, and by suppression of sympathetic outflow from the central nervous system (40, 56, 92). ANF also lowers the activation threshold of vagal afferents, thereby suppressing the reflex tachycardia and vasoconstriction that normally accompany decreased preload, ensuring a sustained decrease in mean arterial pressure (67).

vii). ***Effects of ANF on the Kidney***

The kidney is another major site of ANF activity, where it increases natriuresis and diuresis through both hemodynamic and tubular actions. ANF increases glomerular filtration rate (GFR) by increasing glomerular capillary hydrostatic pressure, a result of afferent arteriolar dilation and efferent arteriolar constriction (72), with the overall effect being no change in (39)total renal blood flow (68). The alterations in renal hemodynamics do not last as long as the tubular natriuretic effects of ANF, suggesting that the vascular changes are short-term responses to hypervolemia (36).

Plasma concentrations of ANF that do not affect glomerular filtration rate are still able to cause natriuresis, demonstrating that the peptide also has direct tubular

activity. The most important tubular effect of ANF is the production of cGMP in the inner medullary collecting duct, which blocks Na^+ absorption (107). ANF also inhibits angiotensin-II stimulated sodium and water transport in the proximal convoluted tubule (49). ANF antagonizes the activity of vasopressin in the cortical collecting ducts by inhibiting tubular water transport (35). ANF infusion markedly inhibits renin and aldosterone secretion, which results in decreased plasma levels of vasopressin (11). The decrease in aldosterone secretion results from the decrease in plasma renin activity and by the direct effects of ANF on aldosterone synthesis inhibition within the adrenal glomerulosa. ANF also antagonizes all the known effects of angiotensin-II including vasoconstriction, vascular smooth muscle proliferation and proximal sodium reabsorption (11, 113).

C. ANF and the Spleen

i). *Background*

Exogenously administered ANF causes an increase in hematocrit and a decrease in plasma volume, which cannot be accounted for by urinary losses (2, 31). We have previously shown that these responses are abolished by splenectomy, and that ANF causes a sustained increase in the hematocrit of blood as it passes through the spleen (57). This is a spleen specific response, since acute hypervolemia, which results in the release of ANF, increases the hematocrit of blood as blood passes through the spleen but had no effect on hindquarter hematocrit (58). Since the rat spleen is non-contractile and has virtually no blood

storage capacity (87), we proposed that the increase in hematocrit does not originate from the expulsion of erythrocytes from splenic reservoirs. Moreover, further experiments revealed splenic venous outflow to be *lower* than splenic arterial inflow. We concluded from these results that there is a significant (~25%) efflux of fluid from the splenic vasculature. This fluid does not remain within the parenchyma of the spleen, but drains through the splenic lymph duct into the systemic lymphatic system. ANF is also able to increase lymphatic capacitance and decrease the return of lymphatic fluid into the systemic circulation by inhibiting the contraction of lymphatic vessel smooth muscle (106), thus, elevated plasma(80) ANF would not only cause increased fluid extravasation from the spleen into the lymphatic system but, by increasing lymphatic capacitance and decreasing its return into the vasculature, it would sustain the reduction in plasma volume, thus normalizing the hypervolemic state.

These findings have led us to believe that the spleen is a major site of ANF-induced translocation of fluid out of the intravascular space. ANF could potentially cause this increase in fluid extravasation by altering capillary permeability within the spleen, as it has already been shown at high doses in other tissues (105). However, splenic capillary beds have been proven to have a discontinuous endothelium (99), which makes it unlikely that fluid extravasation results from increased capillary permeability. Furthermore, we have shown that lymph draining from the spleen is iso-oncotic to the plasma, indicating that the capillary beds are freely permeable to plasma proteins (58). Therefore, we have

proposed that changes in splenic fluid extravasation in response to physiological levels of ANF occur, not through changes in capillary permeability, but via alterations in splenic hemodynamics to increase capillary filtration pressure. Such a mechanism would be analogous to the role of ANF in the kidney, where it raises glomerular filtration pressure by impairing venous efflux, thereby increasing glomerular filtration rate (GFR) (36, 69).

ii). *Proposal to Study the Effects of ANF on Splenic Hemodynamics*

While it is known that ANF is able to affect the function of the spleen, the mechanism by which ANF increases fluid efflux from the spleen remains to be elucidated. The present study was designed to determine the mechanism by which ANF increases fluid efflux from the spleen. To this end, we devised a blood-perfused spleen preparation, in which blood flow was held constant while perfusion pressure was monitored. In order to avoid complications arising from the effects of ANF on sympathetic nerve activity (100) and systemic blood pressure (20), the peptide was infused directly into the splenic artery and the spleen was denervated (Figure 3). Application of the double vascular occlusion technique allowed us to determine changes in hemodynamic functions such as capillary pressure, and pre- and post-capillary resistance within the spleen during ANF infusion (48, 104). Use of this technique enabled us to evaluate rapid changes in capillary hemodynamics induced by various pharmacological or pathophysiological stimuli (104). A similar protocol was repeated in the hindquarters to ensure that that our observations were spleen-specific.

It may be argued that an increase in intrasplenic capillary pressure by ANF does not necessarily cause increased fluid extravasation into the systemic lymphatic system. In order to verify that a rise in capillary pressure did indeed increase fluid extravasation, transit time flow probes were used to measure the effect of ANF on splenic venous outflow compared to arterial inflow (26).

We also wished to determine whether ANF-induced changes fluid extravasation could be mimicked by physically increasing intrasplenic capillary pressure. To this end, the portal vein, into which the splenic vein drains, was partially occluded (27, 81). This increased the resistance of splenic venous outflow, thereby elevating splenic capillary pressure. Transit-time flow probes were used to confirm that the increase in splenic capillary pressure induced by portal hypertension did result in increased fluid extravasation from the spleen. An increase in arterio-venous difference in blood flow during portal hypertension allowed us to demonstrate that mechanically raising capillary pressure mimics the effects of ANF in the spleen, thereby causing increased fluid extravasation.

iii). *Proposal to Characterize ANF-induced Vasoconstriction*

Since we found that ANF increases splenic capillary pressure by increasing post-capillary resistance, we therefore sought to characterize the NPR-subtype responsible for the vasoconstrictive actions of ANF in the spleen. To this end, we used the isolated, blood-perfused spleen preparation described earlier, in which

blood flow was held constant while perfusion pressure was monitored. In order to isolate the specific NPR-subtype responsible for vasoconstriction, we infused ANF, the ANF antagonist A71915, and NPR_C-specific ligand C-ANF, directly into the isolated, blood-perfused spleen (39). We then applied the double vascular occlusion technique in order to measure changes in hemodynamic parameters, such as capillary pressure (P_C) and post-capillary resistance (R_V) within the spleen during peptide infusion (16, 104).

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

A. Animals and Surgery

i). *Animal Use*

The experiments described in this thesis were examined by University of Alberta Health Sciences and Animal Welfare Committee, and found to be in compliance with the guidelines issued by the Canada Council on Animal Welfare. At the completion of the studies, all animals were killed with an anesthetic overdose (0.3 ml I.V., Euthanyl, MTC Pharmaceuticals, Cambridge, Ontario, Canada).

Male Long-Evans rats (450-650 g body weight) were obtained from Eastern Canada (Charles River, St. Foy, Quebec, Canada). Animals were held in the University Animal Facility for at least one week prior to surgical or experimental procedures, exposed to light of 12/12-hour cycle, in a humidity and temperature controlled environment, and maintained on a 0.3% sodium diet and water *ad libitum*.

ii). *Anesthesia*

Anesthesia was induced with isoflurane (2.5%; IsoFlo™, Abbott Laboratories, U.S.A), an inhalable anesthetic. The administration of isoflurane continued until the femoral vein was cannulated, at which time Somnotol (sodium pentobarbital, 65 mg ml^{-1}; MTC Pharmaceuticals, Cambridge, Ontario, Canada) could be

infused I.V. (50 mg kg^{-1}). Inactin (ethyl-(1-methyl-propyl)-malonyl-thio urea; 80 mg kg^{-1}, S.C.; BYK, Germany), given at the end of surgery, maintained the rat under a moderate surgical plane of anesthesia (no paw-pinch response) for the duration of the experiment.

B. Blood-perfused spleen

i). *Abdominal Vasculature*

In order to ensure that the splenic artery and vein supplied and drained only the spleen, all branches running from the splenic vessels to the pancreas, stomach and other surrounding tissues were ligated and divided (Figure 3). We have previously confirmed by injecting dye into the splenic artery that this procedure ensures vascular isolation of the spleen (26).

ii). *Surgery*

Once the rats were anesthetized, they were shaved over cannula insertion points as well as along the back, ensuring an adequate connection to the electric cauterizer plate placed underneath. During surgery, the rats were placed on an isothermal heating pad to maintain body temperature within the normal physiological range of 38-39°C.

Femoral Cannulae: A 1.5 cm long medial incision was made along the upper left leg, and then the fatty tissue and the adductor longus muscles were bluntly dissected to expose the femoral artery and vein. The femoral nerve was dissected

away from the femoral artery and vein and then the two vessels were carefully separated from each other. The femoral vein was cannulated using silastic tubing (0.51 mm i.d., 0.94 mm o.d.; Dow Corning, USA). Once the cannula was inserted, it was proximally advanced up the femoral vein approximately one inch and secured in place. The femoral artery was cannulated with PE-50 tubing (0.58 mm i.d., 0.965 mm, o.d.; Intramedic, USA) using a similar protocol as the femoral vein. Mean arterial pressure was monitored via the femoral artery, and Somnotol was administered through the femoral vein. The venous line was also used to infuse isotonic saline (3 ml hr^{-1}) to maintain adequate hydration of the animal throughout the duration of the experiment.

Carotid Cannula: A 2 cm midline incision was made in the ventral skin of the neck and the fatty tissue and underlying muscle were bluntly dissected and retracted to expose the right common carotid artery. Once cleared, the carotid artery was occlusively cannulated using PE-90 (0.86 mm i.d., 1.27 mm o.d.) tubing. The purpose of the carotid line was to provide the source of oxygenated-blood for the splenic perfusion.

Gastric Cannulae: A ventral midline abdominal incision was made from the xiphisternum along the linea alba to the lower part of the abdomen using the electric cauterizer. The spleen was carefully cleared from the stomach and replaced in its natural position in the abdominal cavity. The stomach was cleared of all connective tissue, wrapped in saline soaked gauze, and then retracted out of

the abdominal cavity and placed on top of the thoracic cavity. This exposed the left gastric artery and vein, which were to be used to access the splenic artery and vein (Figure 3). The left gastric artery and vein were carefully cleared using fine Moria forceps, with great care being taken to handle the vessels as little as possible, since they are capable of extreme vasoconstriction. The gastric artery was cannulated with drawn out PE-50 tubing (0.58 mm i.d., 0.965 mm, o.d.), while the gastric vein was cannulated with Micro-Renethane tubing (0.30 mm i.d., 0.64 m o.d.; Braintree, USA). The gastric artery cannula was connected, via a 3-way adapter, to a pressure transducer (splenic perfusion pressure), and to the peristaltic pump. The venous cannula was advanced to the junction of the gastric vein and the splenic vein, and was connected to a pressure transducer in order to monitor the venous pressure of the blood-perfused spleen.

When the surgery was completed, splenic perfusion was initiated. The splenic perfusion consisted of oxygenated blood taken from the carotid artery, and perfused into the splenic artery via the peristaltic perfusion pump (1.0 ml min^{-1}). Systemic pressure and splenic arterial and venous perfusion pressures were monitored online using a data acquisition board (D1-400, DATAQ Instruments, Akron, Ohio, USA) throughout the duration of the experiment and recorded using DATAQ's own software (WINDAQ).

C. Blood-Perfused Hindquarters

In order to ensure that any results from our splenic perfusions were spleen-specific, we repeated the experiment on the rat hindquarters, following a similar protocol to that used for the spleen. However, the following changes were made from the blood perfused spleen protocol:

The abdominal aorta was cannulated in two locations. The first cannula (PE-50; 0.58 mm i.d., 0.965 mm, o.d) was inserted non-occlusively into the abdominal aorta towards the heart until it was just below the left renal artery, in order to measure systemic blood pressure. A second line (PE-90; 0.86 mm i.d., 1.27 mm o.d.) was inserted just below the first cannula, and caudally advanced until it was just above the bifurcation of the abdominal aorta. This second line served to deliver oxygenated blood to the hindquarter (3.0 ml min^{-1}) from the carotid artery; it was also used to measure arterial perfusion pressure to the isolated vascular bed. The inferior vena cava was cannulated with silastic tubing (0.51mm i.d., 0.94 mm o.d.), and was connected to a pressure transducer in order to measure venous perfusion pressure.

D. Double Occlusion Technique

In both the blood-perfused spleen and the blood-perfused hindquarter experiments, capillary pressure was determined using the double vascular occlusion technique (104). After stabilization, both inflow and outflow cannulas

were simultaneously occluded. Arterial pressure (P_A) and venous pressure (P_V) equilibrated rapidly to a value reflective of capillary pressure (P_C) (Figure 4). If P_A and P_V did not exactly equilibrate to the same pressure upon double occlusion, then the mean of both pressures was determined and was defined as P_C (16). Results of previous studies have shown that capillary pressures measured by the double vascular occlusion are equivalent to those measured by other classical means, such as the micro-puncture technique (41, 47).

The circulation of blood through the spleen and the rat hindquarters can be represented by a simple linear model where P_A is separated from P_C by a pre-capillary resistance (R_A), and P_C is separated from P_V by a post-capillary resistance (R_V). The pre-capillary and post-capillary resistances are calculated using the following equations;

$$R_A = (P_A - P_C) / Q$$

$$R_V = (P_C - P_V) / Q$$

where Q is equal to flow rate (ml min^{-1}) (16).

E. Experiment 1 Protocol

Separate groups of animals were used for the measurement of ANF-induced changes in splenic and hindquarter hemodynamics, of ANF-induced changes in splenic blood flow and of the hemodynamic consequences of portal hypertension.

i). *Spleen*

Once the perfusion of the spleen (1.0 ml min^{-1}) had started, heparin (0.15 ml; 10,000 i.u. ml^{-1}) was injected I.V. in order to prevent thrombus formation within the perfusion tubing. The animals were then allowed to stabilize for a period of 30 min before ANF infusion was initiated into the splenic artery at a rate of 50 µl min^{-1} and at doses of 1, 5, 20, 60, 180 ng min^{-1}. Double vascular occlusions were conducted at 5, 10 and 20 min after ANF infusion began. The capillary pressure is reported as the mean of these three readings, as there was no significant difference between them. The double occlusion was performed by simultaneously tightening a snare placed around the splenic vein while the perfusion pump was stopped and the tubing clamped, so that arterial inflow was blocked for a period of ~5 seconds (47). Control animals, which had been implanted with the same cannulae and treated in the same manner as the experimental animals, were infused with saline and subjected to the same protocol.

ii). *Hindquarters*

Once the hindquarter perfusion had started (3.0 ml min^{-1}), heparin (0.15 ml; 10,000 i.u. ml^{-1}) was injected I.V. in order to prevent thrombus formation within the perfusion tubing. The animals were then allowed to stabilize for a period of 30 min before ANF infusion was initiated into the hindquarters at a rate of 50 ul min^{-1} and at doses of 5, 20, and 60 ng min^{-1}. Double vascular occlusions were conducted at 5, 10 and 20 min after ANF infusion began. Similar to the spleen, the capillary pressure is reported as the mean of these three readings, as there was

no significant difference between them. The double occlusion was performed by simultaneously tightening a snare placed around the inferior vena cava just below the renal veins while the perfusion pump was stopped and the tubing clamped, so that arterial inflow was blocked for a period of ~5 seconds. Control animals, which had been implanted with the same cannulae and treated in the same manner as the experimental animals, were infused with saline and subjected to the same protocol.

iii). *Blood Flow*

In order to verify that the ANF-induced alterations in capillary pressure resulted in the transfer of fluid from the vascular compartment to the lymphatic system, a transit-time flow probe (R1; Transonic, New York, USA) was placed around the splenic vein. Great care had to be taken when clearing the splenic vein from the pancreatic tissue as excessive contact with the highly sensitive splenic vein resulted in excessive vasoconstriction and abdominal bleeding (26, 33). The spleens were perfused in a similar fashion as previously described at a constant flow rate of 1 ml min^{-1} and ANF (20 ng min^{-1}) was infused into the spleen for a period of 30 min.

iv). *Portal Hypertension*

The portal vein was partially occluded once the blood-perfused spleen had stabilized. This was achieved by tightening a ligature around the portal vein and a 20-gauge needle; after the ligature was secured, the 20-gauge needle was

removed, leaving the portal vein partially occluded in a consistent manner (27, 81). This caused splenic venous pressure to increase to ~15 mmHg. Splenic hemodynamics were then measured using the double occlusion technique.

In order to verify that the mechanical increase in capillary pressure resulted in increased fluid extravasation from the spleen, transit time flow probes (R1; Transonic, New York, USA) were placed around the splenic artery and vein; the flow differential between arterial inflow and outflow indicated the rate of fluid extravasation from the splenic vasculature. Due to technical difficulties, these experiments were done in physiologically intact spleens perfused, not through a pump, but via the normal arterial supply (aorta, celiac artery, splenic artery). The portal vein was partially occluded for a 2 min period, over which time the inflow and outflow rates were averaged and compared to baseline values in the same animal (27, 81).

F. Experimental 2 Protocol

i). *ANF*

After 30 minutes of stabilization, rat ANF (99-126) was infused into the spleen at two doses (20 and 180 ng min^{-1}; n = 4, n = 3 respectively). Double vascular occlusions were conducted after 5, 10 and 20 min of ANF infusion. Control animals, which had been implanted with the same cannulae and treated in the same manner as the experimental animals, were infused with saline.

ii). *Effects of C-ANF (4-23)*

C-ANF (Des [Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]-ANF 4-23-NH_2; rat), an NPR_C-specific agonist (70), was infused in a similar fashion as ANF at concentrations of 20 and 200 ng min^{-1} (n = 3 for both). The double occlusion was then performed and splenic capillary pressure and vascular resistance were measured.

iii). *Effects of A71915 and ANF*

A71915 (Arg^6, β-cyclohexyl-Ala^8, D-Tic^{16}, Arg^{17}, Cys^{18})-ANF (6-18)-NH_2; rat), an ANF specific antagonist (110), was infused into the spleen at two concentrations (16 and 160 ng min^{-1}; n = 4 for both) in order to determine if it had any intrinsic activity within the splenic vasculature. A71915 was then combined with ANF and infused into the spleen at the following doses:

1. ANF (20 ng min^{-1}) and A71915 (16 ng min^{-1}) (n = 4)
2. ANF (20 ng min^{-1}) and A71915 (160 ng min^{-1}) (n = 4)
3. ANF (200 ng min^{-1}) and A71915 (16 ng min^{-1}) (n = 4)
4. ANF (200 ng min^{-1}) and A71915 (160 ng min^{-1}) (n = 4)

G. Statistical analysis

The significance of alterations in capillary pressure and the pre- and post-capillary resistances of the rat spleen and hindquarters were assessed by One-Way ANOVA followed by Student-Neuman-Keuls *post hoc* test for multiple comparisons. The level of statistical significance was defined at $P < 0.05$.

The significance of ANF-induced alterations in blood flow in the perfused spleen, and the portal hypertensive group were assessed by Student's t-test for paired data, since both control and experimental readings were taken in the same animals. Significance was defined at $P < 0.05$, with data are expressed as mean values $\pm$ S.E.M.

CHAPTER III

RESULTS

CHAPTER III

RESULTS

There was a dose-dependent increase in splenic capillary pressure in response to ANF concentrations of 0, 1, 5, 20, 60, 180 ng min^{-1} (n = 8, 3, 5, 5, 6, and 3 respectively) (Figure 5). There was no significant change in pre-capillary resistance (R_A) in the ANF-infused groups compared to the control animals ($P > 0.05$) (Figure 6). However, post-capillary resistance (R_V) of the experimental groups rose significantly in a dose-dependent manner (Figure 7).

The highest dose of ANF (180 ng min^{-1}) did not significantly alter mean arterial pressure. Basal pressure was 104.9 $\pm$ 4.5 mmHg. At the end of 20 min infusion of ANF (180 ng min^{-1}) it was 104.9 $\pm$ 2.2 mmHg ($P > 0.05$).

In the hindquarters, even the highest dose of ANF (60 ng min^{-1}) did not alter capillary pressure compared to the saline-infused control animals (ANF 9.2 $\pm$ 0.4; saline 9.6 $\pm$ 0.1 mmHg; n = 4 $P > 0.05$) (Figure 5). Nor were there any significant changes in pre- and post-capillary resistance (Figure 6, 7 respectively).

In the isolated, blood-perfused spleen, intrasplenic infusions of physiological levels of ANF (20 ng min^{-1}; n = 3) for 30 min resulted in a significant drop in venous blood flow from control values of 0.9 $\pm$ 0.1 ml min^{-1} to 0.7 $\pm$ 0.1 ml min^{-1} ($P < 0.05$) in the absence of any change in splenic inflow (1 ml min^{-1}). The

arterio-venous difference increased from 0.1 ± 0.1 to 0.3 ± 0.1 ml min^{-1}. This represents a total fluid efflux of 9 ml from the spleen over the 30 min period of ANF infusion.

In the isolated, blood perfused spleen, an increase in portal pressure (and therefore splenic outflow pressure) from 3.1 ± 0.5 mmHg to 15.0 mmHg caused intrasplenic capillary pressure to increase from 11.2 ± 0.1 mmHg to 22.4 ± 0.2 mmHg (n = 4) ($P < 0.05$) (Figure 8).

In the physiologically intact spleen, splenic arterial blood flow did not change after the portal vein was ligated (2.1 ± 0.3 vs. 1.9 ± 0.4 ml min^{-1}). However venous blood flow markedly fell (baseline, 1.6 ± 0.2 mmHg vs. portal hypertension, 0.7 ± 0.3 ml min-1), resulting in a sustained increase in the arterio-venous difference in blood flow (+1.4 ± 0.3 ml min^{-1}).

ANF dose-dependently increased splenic capillary pressure (P_C) from control values of 11.5 ± 0.5 mmHg to 12.4 ± 0.5 and 14.9 ± 0.9 mmHg, at doses of 20 and 200 ng min^{-1} respectively ($P < 0.05$) (Figure 9). ANF also increased post-capillary resistance (R_V) at both the low and high doses (control: 8.4 ± 0.4 mmHg ml^{-1}; low-dose ANF: 9.2 ± 0.4 mmHg ml^{-1}; high-dose ANF 11.3 ± 0.6 mmHg ml^{-1}).

Splenic infusion of C-ANF (20 and 200 ng min^{-1}), a NPR_C specific agonist, did not significantly alter either capillary pressure or post-capillary resistance in comparison to control groups (Figure 10) ($P > 0.05$).

The ANF antagonist A71915 did not demonstrate any intrinsic activity; as splenic P_C and R_V were unchanged for the duration of both low (16 ng min^{-1}) and high dose (160 ng min^{-1}) infusions (Figure 11).

However, A71915 inhibited ANF-mediated alterations in splenic hemodynamics when combined with low doses of ANF (20 ng ml^{-1}) (Figure 12). When high-doses of ANF were infused into the spleen (200 ng ml^{-1}), correspondingly higher doses of A71915 (160 ng min^{-1}) blocked the vascular effects of ANF, while lower doses (16 ng min^{-1}) attenuated the ANF-induced increase in post-capillary resistance and capillary pressure.

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

A. Hemodynamic Effects of ANF

The results of this study are consistent with our proposal that ANF increases fluid extravasation from the spleen through changes in intrasplenic capillary hydrostatic pressure. ANF was found to dose-dependently increase capillary pressure in the spleen (Figure 5). While it did not significantly alter pre-capillary resistance (Figure 6), ANF did increase post-capillary resistance within the spleen (Figure 7). This selective constriction of the post-capillary vasculature of the spleen resulted in the increased capillary hydrostatic pressure and subsequent extravasation of fluid from the plasma to the lymphatic system.

Mean arterial blood pressure was stable for the duration of the perfusions. The spleen was also denervated. Therefore it can be assumed that there were no changes in hormonal or neural input to the spleen from the rest of the body. Splenic blood flow was held constant throughout the experiment by the peristaltic perfusion pump, which prevented any alterations in the rate of blood flowing into the spleen. Therefore, the hemodynamic changes observed in the spleen in our experiments were solely due to ANF-induced alterations within the splenic vascular bed.

The effects of ANF on splenic hemodynamics and fluid extravasation were tissue specific, since there were no significant alterations in capillary pressure or vascular resistance in the perfused hindquarter groups under the same conditions. These results are consistent with previous findings in this lab demonstrating that in hypervolemia, a condition where ANF is endogenously released, venous hematocrit from the spleen was increased, while there was no change in the venous hematocrit of blood passing through the hindquarters (58).

In order to demonstrate that the ANF-induced increase in capillary pressure and post-capillary resistance was accompanied by fluid extravasation, venous outflow was measured before and after the administration of a physiological dose of ANF (20 ng min^{-1}) into the spleen. It is important to emphasize that that, unlike the dog, the rat spleen is not contractile and is unable to act as a blood or lymph storage organ (87, 97). Therefore, the effects of ANF on splenic hemodynamics are considered to be independent of blood sequestration into the spleen. Since blood flow into the spleen was held constant during the experiment by the perfusion pump, the alterations in venous outflow represent the transfer of fluid from the vasculature and into the systemic lymphatic system. Venous blood flow dropped significantly (0.9 ± 0.1 ml min^{-1} to 0.7 ± 0.1 ml min^{-1}) during the infusion of ANF, proving that the increase in intrasplenic capillary pressure correlates with the transfer of fluid out of the vasculature. The total volume of fluid translocated from the blood into the lymphatic system over the 30 min infusion of ANF was calculated to be 9 ml, many times the total intrasplenic volume (splenic weight

~1.0 g). This provides further proof that the fluid extravasated from the spleen is transferred to another storage site, since it is impossible for such a large volume of fluid to be accommodated within a non-compliant organ.

The existence of a deep splenic lymphatic system has been confirmed by Pellas et al. (84). Therefore it is reasonable to suggest that fluid from the splenic vasculature is continuously drained into the lymphatic system, and that the rate of fluid extravasation is increased by ANF. Increased fluid extravasation by ANF would not, by itself, reduce blood volume. However, ANF is also able to increase lymphatic capacitance and decrease the return of lymphatic fluid into the systemic circulation by inhibiting the contraction of lymphatic vessel smooth muscle (8, 80, 106). Therefore, ANF not only increases fluid extravasation from the spleen into the lymphatic system but, by increasing lymphatic capacitance and decreasing its return into the vasculature, it is also able to sustain the decrease in plasma volume, thus correcting the surfeit in intravascular volume.

It may be argued that, despite its effects on the splenic microvasculature, ANF may alter the rate of fluid extravasation from the spleen through a mechanism that is completely independent of its hemodynamic effects. The purpose of the portal hypertension experiments was to demonstrate that increases in splenic capillary pressure are directly correlated with increased fluid extravasation. Since the splenic venous outflow drains into the portal vein, the resistance of splenic venous outflow would be increased by inducing portal hypertension. This caused a

significant increase in intrasplenic capillary pressure (Figure 8), which was associated with increased fluid efflux from the splenic circulation. This confirms that increased splenic capillary pressure, whether mechanically stimulated by portal hypertension or hormonally stimulated by ANF, directly results in increased fluid extravasation.

However, the question remains as to where such a large volume of fluid is transferred. Lymphatic fluid from the splanchnic circulation generally flows through a series of lymphatic vessels and ducts, ultimately reaching the thoracic duct (64, 102). While most of the deep lymphatic vessels of the spleen empty into the thoracic duct, a significant portion of the lymphatic fluid from the spleen drains directly into the portal vein (55). Upon partial occlusion of the portal vein, lymphatic fluid was observed to back up into the splenic vascular arcade. Since this same phenomenon has been observed after volume loading (58), it is possible that the spleen has accessory lymphatic drainage pathways to accommodate the very high rate of lymph flow from this organ.

Summary

We demonstrated that ANF, when infused directly into the spleen, produces a dose-dependent increase in splenic post-capillary vasoconstriction, which results in a rise in intrasplenic capillary pressure. The increase in capillary pressure elevates intrasplenic hydrostatic pressure, thus causing extravasation of iso-oncotic fluid from the spleen into the lymphatic system. However, the

mechanism underlying the novel constrictor activity of ANF remained to be elucidated. In order to isolate the natriuretic peptide receptor-subtype responsible for mediating vasoconstriction, we studied the effects of ANF, the ANF antagonist A71915, and NPR_C-specific ligand C-ANF, in the same isolated, blood-perfused setting.

B. Role of Natriuretic Peptide Receptors

The results of this study are consistent with our previous work showing that ANF causes a dose-dependent increase in splenic capillary pressure and post-capillary resistance. We were also able to demonstrate that NPR_A is responsible for mediating the ANF-induced alterations in splenic hemodynamics, while the activation of NPR_C appears to function independently of the vasoconstrictive response to ANF in the spleen. While the binding of ANF to NPR_A results in the production of cGMP and typically, vasodilation, the novel vasoconstrictor action of ANF has been found to be mediated by NPR_A in the renal vasculature as well (39).

ANF has been proven to bind with high specificity to NPR_A and NPR_C at the dose used in this study (7, 15). Activation of NPR_A stimulates the production of cGMP and is believed to be the primary mediator of the cardiovascular effects of ANF (61). In contrast to the well-documented ability of ANF to cause vasodilation

through the cGMP-dependent signaling cascade, the mechanism by which ANF mediates vasoconstriction remains unclear.

Classically, NPR_C has been characterized as a clearance receptor, accepting all natriuretic peptides with high affinity (70). Recent research has revealed that activation of NPR_C also results in the inhibition of adenylyl cyclase and mitogen-activated protein kinase (MAPK) activity stimulated by endothelin and platelet-derived growth factor. This eventually results in the inhibition of DNA synthesis, and the anti-mitogenic properties associated with ANF (6, 50, 52). Due to the new signaling pathways discovered for NPR_C, we initially thought it possible that the activation of NPR_C may stimulate the release of an undiscovered second messenger capable of generating the ANF-induced vasoconstriction.

ANF (20 and 200 ng min^{-1}) caused a dose-dependent increase in splenic post-capillary resistance and capillary pressure (Figure 9). The paradoxical vasoconstriction we observed is not without support in the literature, as ANF has been shown to induce vasoconstriction in a variety of other vascular beds such as the mesenteric and renal vasculature (72, 94, 115, 117). It is also important to note that the vasoconstrictive activity of ANF has not only been observed in rats, but in many other animal models such as the dog and monkey (95). Indeed, the splenic vessels reacted in remarkably similar fashion to the renal vasculature, where ANF induces vasoconstriction selectively in the efferent arterioles, thus increasing glomerular filtration pressure and glomerular filtration rate (39).

C-ANF is an NPR_C-specific agonist that competes effectively with biologically active ANF for binding sites on NPR_C. It has been proven to have no agonist or antagonist action on the generation of cGMP in vascular smooth muscle cells and endothelial cells *in vitro* (53, 70). C-ANF failed to constrict post-capillary vessels or increase capillary pressure within the spleen. Due to the specificity of C-ANF binding to only NPR_C and the fact that it has no intrinsic activity on cGMP production, the vascular activity of ANF within the spleen is not likely to be mediated by the NPR_C.

A71915 is a ring-deleted analogue of ANF that has the ability to inhibit ANF-stimulated cGMP production. A71915 belongs to a class of compounds that were developed to specifically inhibit ANF-mediated cGMP production (110). The efficacy of A71915 as an ANF-inhibitor has been verified using a variety of different cell lines, such as cultured neuroblastoma NB-OK-1 cells that exclusively express NPR_A (32). In that study, A71915 demonstrated a strong ability to inhibit ^{125}I-ANF binding and significantly suppressed ANF-stimulated cGMP production, causing a rightward 2.8-log shift of the ANP dose-response curve in the cGMP assays.

A71915 was used to investigate whether the vasoconstrictive actions of ANF with the spleen are mediated by the activation of NPR_A. In the blood-perfused spleen, A71915 alone did not demonstrate any intrinsic activity, as was evident by the

lack of changes in vascular resistance or capillary pressure. However, A71915 completely inhibited the ANF-mediated alterations in splenic hemodynamics when combined with low doses of ANF (20 ng min^{-1}). The effects of high doses of ANF (200 ng min^{-1}) on the splenic vasculature were also inhibited when correspondingly high doses of A71915 were simultaneously infused into the spleen. The ability of A71915 to inhibit the effects of ANF within the spleen further suggests that ANF alters splenic hemodynamics through the activation of NPR_A and the subsequent formation of cGMP.

Summary

In conclusion, our results have demonstrated that NPR_A is responsible for mediating the ANF-induced alterations in splenic hemodynamics, while the activation of NPR_C appears to function independently of the vasoconstrictive response to ANF in the spleen. However, the manner in which NPR_A modulates splenic vasoconstriction remains unclear.

C. Mechanisms of Constriction

Despite the generally accepted view that ANF acts primarily as a vasodilator, evidence from *in vivo* studies indicate that exogenous ANF administration causes vasoconstriction in a variety of vascular beds throughout the body, such as the mesenteric, renal and splenic vasculatures (95, 115). It is important to note that the vasoconstriction observed in these studies has been proven to be independent of the renin-angiotensin system, vasopressin and sympathetic output (37, 73, 116).

The vasoconstrictive actions of ANF in these experiments occurred within the range of physiologically circulating ANF concentrations (1 - 50 pmol) (15, 96). When significantly higher doses of ANF were infused, these vascular beds demonstrated a transient vasodilator effect, but this was followed by a prolonged vasoconstrictor response (95).

While information on the constrictive actions of ANF is sorely lacking, there is even less information regarding its mechanism. Our results have demonstrated that ANF increases post-capillary resistance in splenic vessels through the activation of NPR_A. Similar conclusions have also been made by researchers studying the constrictive effects of ANF on renal efferent arterioles (39). However, this theory is a paradox, as NPR_A is linked to membrane bound, particulate guanylyl cyclase, and the subsequent activation of it results in cGMP formation and typically vasodilation (75).

All of the *in vivo* evidence that ANF causes vasoconstriction rather than vasorelaxation has largely been ignored by the research community, primarily because of the large amount of *in vitro* evidence that ANF acts as a vasodilator through the stimulation of cGMP production. However, there is an increasing body of evidence suggesting that cGMP is able to cause vasoconstriction. For instance, cGMP has been found to stimulate Ca^{2+} release from the sarcoplasmic reticulmn in gastrointestinal smooth muscle cells after blockade of cGMP-dependent protein kinase G (PKG) (77). This novel study also demonstrated that

the cGMP-mediated Ca^{2+} release was additive to the Ca^{2+} released via IP_3, indicating that cGMP mobilizes a distinct source of Ca^{2+}. While these findings appear to be significant, the ability of cGMP to mediate Ca^{2+} release and subsequently vasoconstriction independently of PKG *in vivo* remains to be established.

The addition of a small amount of hemolysate to the buffer solution of an isolated lung preparation has been found to paradoxically cause a strong vasoconstriction, rather than a vasodilator response, to the infusion of 8-Br-cGMP, a cGMP analogue (108). While this response was inhibited by a variety of Ca^{2+} antagonists, as well as the protein phosphatase inhibitor okadaic acid, the authors were unable to identify the hemolysate substrate that caused the pulmonary vasculature to constrict in response to nominally vasodilating stimuli, namely cGMP. This study is of particular relevance to our research on the effects of ANF on the splenic vasculature. Since the spleen plays an important role in the clearance of old or defective blood cells (111), the presence of hemolysate that results from erythrocyte breakdown could potentially cause the vasoconstrictive response to ANF we observed. This hypothesis is supported by the fact that there were no alterations seen in the resistance of pre-capillary vessels upstream of the spleen, while the resistance of post-capillary vessels, which are exposed to the hemolysate, was markedly increased. While splenic hemolysate may contribute to the vasoconstrictive actions of ANF in the spleen, it is unlikely that it is the only mechanism responsible. Indeed, recent studies from our laboratory have

demonstrated that isolated splenic vessels constrict in response to ANF, even though it is a buffer perfused system and hemolysate is absent.

Given that there is no *in vivo* evidence that cGMP acts directly on blood vessels to cause vasoconstriction, it is much more likely that the ANF-induced is caused by the release of a secondary constrictor agent. Since the spleen is predominantly innervated with sympathetic nerves (4, 12), catecholamines are a potential candidate to mediate the constrictive actions of ANF. It is more likely that ANF stimulates the release of catecholamines by acting on nerve terminals rather than on an upstream target, since mesenteric vasoconstriction caused by ANF is largely prevented by β-adrenergic receptor inhibition, but not by autonomic ganglion blockade (116).

ANF could also mediate its vasoconstrictive actions through the release of an autocrine or paracrine hormone. ANF-induced renal efferent arteriole vasoconstriction is abolished after either angiotensin-converting enzyme (ACE) inhibition or endothelin $ET_{A/B}$ receptor blockade (38). These results suggest that the stimulation of NPR_A by ANF could increase local angiotensin-II or endothelin-1 concentrations, thereby causing vasoconstriction of the splenic vasculature. While these findings have shed light on the paradoxical ability of ANF to cause vasoconstriction, a great deal of research remains to be completed before the mechanism underlying this phenomenon is clarified.

D. Experimental Design

i). *Animal Model*

Although recent work has made substantial contributions to our knowledge of splenic function, there are several problems that impose limitations on the study of the spleen. Due to the considerable interspecies variability in the structure and function of the spleen, caution must be applied when extrapolating data available from animal studies into a clinical setting. For instance, animal models such as the dog and horse possess contractile spleens that are capable of storing large quantities of high hematocrit blood, while animal models like the rat have virtually no intrasplenic blood storage capacity at all. Since the human spleen has negligible blood storage capabilities (30-50 ml) (30, 46), it is very difficult to characterize it's ability to control blood volume using an animal model who regulates splenic volume homeostasis in a completely different manner (i.e. dog, horse, cat etc.). Furthermore, it would be difficult to study splenic hemodynamics in an animal with a high-capacitance, contractile spleen, as flow may be complicated by the transient expulsion of high hematocrit blood.

In light of the difficulties posed by the species-dependent differences in splenic function, the rat was chosen as our experimental animal model to investigate the spleen's ability of to control fluid volume homeostasis (87). The rational behind using the rat is that it is both structurally and physiologically similar to the human spleen, which has limited contractile ability and limited blood storage capacity (4). While the rat spleen is not able to store blood, it is still able to filter off cell-

free fluid from the blood to the lymphatic system. Previous experiments from our lab have confirmed this ability of the spleen by showing that 1) splenic venous hematocrit is consistently higher than splenic arterial hematocrit, and 2) splenic venous blood flow is significantly lower than arterial blood flow (58), even under normal physiological conditions.

The spleen has been proven to increase the rate of fluid extravasation in response to acute volume loading and atrial stretch (26). Since the rat spleen is unable to store blood (87), the increase in hematocrit does not originate from the expulsion of erythrocytes from splenic reservoirs, but from a reduction in plasma volume. Even if the rat spleen *did* act as a blood volume reservoir, the expulsion of high hematocrit blood during conditions capable of simulating hypervolemia (i.e. volume expansion, atrial stretch, ANF etc.) would be physiologically inappropriate because it would add more blood to an already overexpanded intravascular compartment.

There is no endothelial continuity between the terminal arterioles and the proximal veins in both the rat and human spleen (45). This makes the spleen freely permeable to plasma proteins, which has been confirmed by the iso-oncotic nature of lymph fluid draining from the splenic vasculature (58). Consequently, the main driving force behind the extravasation of fluid from the spleen *must* be capillary hydrostatic pressure, as any potential changes in capillary permeability

would have little effect considering it is freely permeable under normal physiological conditions.

ii). ***Blood Perfused Spleen***

Studies of splenic hemodynamics have been hampered by the delicate nature of this organ. Because of this, a great deal of splenic research has been conducted *in vitro*. Considering the significance of the neural and hormonal control of splenic function, it is questionable whether surgically removed spleens are capable of allowing splenic hemodynamics to be accurately studied. In order to circumvent the problems associated with *in vitro* models, we used an intact, blood-perfused preparation to study splenic hemodynamics, where oxygenated blood is perfused into an intact spleen, with venous outflow returning normally via the splenic vein into the portal vein.

The benefit of using whole blood as the perfusion medium instead of a crystalloid solution is that it creates a much more physiological environment to study splenic hemodynamics. Blood is able to provide a higher level of tissue oxygenation, leading to superior hemodynamic and metabolic performance of the spleen (85). Blood is also a more physiological perfusate with which to study splenic hemodynamics, since rheological factors such as blood viscosity are are known to affect shear stress, velocity profiles and patterns of flow.

The vessels of the splenic vasculature are highly reactive. Splenic vessels typically become irreversibly constricted in response to any sort of tactile stimulation, such as surgical vessel manipulation (93). Unlike many *in vitro* perfusion models, the preparation used in our experiment did not require extensive surgical manipulation of the spleen and the splenic vasculature, which prevented the occurrence of abnormal hemodynamic responses that could potentially compromise the validity of our data.

iii). ***Double Occlusion***

Capillary hydrostatic pressure is generally considered to be the major driving force behind transvascular fluid filtration. In most whole organ studies, this important determinant of capillary fluid filtration has been estimated using the gravimetric techniques originally described by Papperheimer and Soto-Rivera (63). Application of gravimetric or volumetric techniques requires extensive surgical manipulation and poses significant technical limitations (i.e. the organ must be isolated and continuously weighed), which precludes routine measurement of capillary pressure.

A number of more recent studies have demonstrated a high degree of correlation between the capillary pressure measured by classical methods, such as the isogravimetric technique, and the capillary pressure measured with the double vascular occlusion technique (41, 47, 104). The double vascular occlusion has been proven to consistently yield accurate and extremely rapid estimates of

capillary pressure in a variety of different vascular beds, such as the lung (104), skeletal muscle (63) and the gastro-intestinal tract (43). Unlike isogravimetric or micro-puncture techniques, use of the double occlusion technique allows for the determination of segmental resistance within the organ studied (48). In our experiments, the circulation of blood through the spleen and the rat hindquarters can be represented by a simple linear model where arterial pressure is separated from capillary pressure by a pre-capillary resistance (R_A), and capillary pressure is separated from venous pressure by a post-capillary resistance (R_V) (16).

A major benefit of using the double occlusion technique for measuring capillary pressure is that it is relatively simple, performed rapidly, and does not require extensive surgical manipulations. The capillary pressures measured for each of the experimental groups of our study were consistently within 1 mmHg of each other, reflecting the precision of the double occlusion technique.

iv). ***Drug Administration***

Another critical parameter that must be considered is the effects of the anesthetic agents, which are able to alter base-line parameters of the cardiovascular system such as heart rate, contractility, stroke volume, and total peripheral resistance (18). However, changes in these cardiovascular parameters should have had minimal effects on splenic hemodynamics in our model, as the rate of splenic blood flow was held constant throughout the experiment, independent of any systemic variations. Anesthetic agents are also capable of altering neural

components involved with cardiovascular reflex responses controlled by the central and autonomic nervous systems (18). Since we anesthetized our control animals in the same manner as experimental animals, any potential alterations in neural input to the spleen should have been accounted for and should therefore not have affected our observations. Furthermore, since we denervated the spleen in our experiments, any potential anesthetic-induced alterations in neural activity should not affect the spleen.

Binding of drugs to proteins in the blood stream or to vascular receptors peripheral to the spleen must be considered as another source of error when interpreting experimentally derived data from other studies, as these results may not reflect the potential activity of the substances when they are released *in vivo* (87). In our experiments, we have made an effort to minimize these errors by infusing ANF and its analogues directly into the spleen. This allowed us to use smaller doses than those normally required for systemic administration, decreasing the likelihood that our observations were complicated by the actions of ANF in the peripheral circulation. Even when the highest dose of ANF was used (200 ng min^{-1}), MAP (a hemodynamic parameter that is reflective of the systemic effects of ANF) remained stable and unchanged from baseline values. Another benefit of infusing our peptides directly into the spleen is that it minimized systemic clearance or degradation (82), which enabled us to accurately conclude that the dose of ANF administered was the dose that reached the spleen.

E. Clinical Relevance

Because of its salutary effects on the cardiovascular system, the therapeutic use of ANF is being studied in patients with a variety of pathophysiological conditions such as congestive heart failure, renal failure, hypertension and cirrhosis (68). The anti-mitogenic properties of ANF may be of particular significance to patients suffering from congestive heart failure or essential hypertension (6). However, the deleterious effects of ANF on the cardiovascular system must also be considered. For example, the cardiovascular pathologies associated with septic shock are possibly related to ANF and the role that ANF plays in regulating fluid volume homeostasis.

Septic Shock

Septic shock is the leading cause of intensive care mortality throughout North America (84). The development of septic shock leads to severe and intractable hypotension coupled with a primary reduction in circulating blood volume, which ultimately leads to circulatory collapse (83). Previous studies have demonstrated that splenectomy is able to blunt the decline in mean arterial pressure (MAP), the increase in hematocrit and the reduction in plasma volume that occurs in intact animals infused with lipopolysaccharide (LPS), a gram-negative bacterial endotoxin used to induce septic shock (9). This study also showed that there is an increase in the arterio-venous blood flow differential across the spleen during septic shock. Since the spleen is not capable of storing high hematocrit blood, the

difference in splenic arterio-venous blood flow is directly associated with the extravasation of iso-oncotic fluid from plasma to the lymphatic system.

Plasma levels of atrial natriuretic factor have been shown to markedly increase during septic shock (1). Since atrial natriuretic factor is an important regulator of fluid volume homeostasis, there may be a connection between the hemodynamic alterations (namely the hypotension and blood volume reduction) that occur during septic shock and the actions of ANF within the spleen. Elevated levels of ANF are able to increase post-capillary resistance and capillary filtration pressure within the spleen, resulting in increased extravasation of fluid from the plasma to the lymphatic system. Therefore, it is possible that pathophysiological increases in plasma ANF levels may contribute to the reduction in blood volume that occurs during septic shock. However, it is not likely that ANF is the sole mediator of fluid extravasation from the spleen during septic shock. Intrasplenic nitric oxide (NO) production is dramatically increased during septic shock and it has been proven that NO is also capable of increasing the rate of splenic fluid extravasation (59). Excessive production of intrasplenic NO, when combined with the increased plasma ANF levels, may thus play an important role in causing the hypovolemia associated with septic shock.

The increased fluid extravasation that we have demonstrated may reflect, not only the role the spleen plays in cardiovascular homeostasis, but also the role the spleen plays in mediating the immune response to blood-borne antigens (i.e. LPS)

(62). For instance, activated T-lymphocytes within the spleen are not directly released into the systemic circulation, but are released into the lymphatic vessels that drain from the spleen (84). Therefore, the ANF-mediated increase in splenic fluid extravasation from the plasma to the lymphatic system would facilitate the mobilization of activated immune cells from the spleen to the site of insult. In support of this line of reasoning, activated macrophages within the spleen have been found to synthesize and release ANF, enabling splenic fluid extravasation to be controlled locally in addition to the effects of ANF released systemically into the blood stream by the heart (101). Research using an ovine model of hyperdynamic sepsis has shown that HS-142-1, a NPR_A-specific antagonist, is able to increase cardiac filling pressure and maintain mean arterial pressure (51). Since our studies have proven that ANF regulates the rate of fluid extravasation from the spleen via NPR_A, specific inhibition of this receptor subtype may be used as a future therapeutic strategy in humans to minimize the excessive loss of plasma volume that occurs during septic shock.

CHAPTER V

CONCLUSION

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During hypervolemia, the active form of ANF, a 28-amino acid peptide hormone, is secreted from storage granules within the atria in response to volume-induced stretch (67). ANF is released into the blood and participates in the renal and cardiovascular responses to acute and chronic volume overload. The ability of ANF to shift fluid from the vasculature to the lymphatic compartment serves as a buffering device to limit plasma volume expansion. By decreasing intravascular plasma volume to normal levels, the atria are no longer stretched, and ANF release is inhibited in a negative feedback manner (69).

The actions of ANF in the spleen are analogous to those seen in the kidney, where ANF causes dilation of the glomerular afferent arteriole and constriction of the efferent arteriole. In the kidney, this increases glomerular capillary hydrostatic pressure, which then exceeds oncotic pressure, thus increasing glomerular filtration pressure and glomerular filtration rate (21, 74). We have demonstrated that, in the spleen, ANF likewise increases post-capillary resistance and capillary pressure, thereby elevating hydrostatic pressure and causing subsequent fluid efflux from the intravascular space. However, unlike the renal glomerulus, the splenic circulation is freely permeable to plasma proteins (58). Since there is no colloid osmotic gradient across the capillary wall to oppose fluid efflux, modest increases in splenic capillary hydraulic pressure are able to induce a significant

increase in fluid extravasation. Therefore capillary hydraulic pressure, although higher than that observed in the hindquarter vasculature, is not elevated to the same extent as seen in the renal circulation (107).

The concept that ANF is able to alter splenic hemodynamics is not without support in the literature. ANF and ANF receptors, as well as their respective gene-transcripts, have been identified in the spleen of a variety of species (42, 65, 65, 101, 101, 109, 109). Despite the fact that the vasodilatory activity of ANF has been thoroughly investigated, the mechanisms underlying its constrictive effects in the mesenteric, renal and splenic vasculatures are still unknown.

Just as extravascular volume is determined by total body sodium (Na^+), intravascular volume is directly dependent on total protein content of the plasma (17). ANF influences renal function through changes of renal hemodynamics and sodium reabsorption. Through the renal regulation of Na^+ excretion, ANF is capable of controlling total *extracellular* fluid volume. However, the renal glomerulus is impermeable to albumin (10). Therefore, the kidney cannot specifically influence intravascular protein content and volume.

In contrast, the spleen is freely permeable to albumin, and ANF can therefore control the efflux of iso-oncotic fluid from the splenic blood into the systemic lymphatic system. ANF is also able to increase lymphatic capacity, and slows the return of lymphatic fluid to the vasculature by inhibiting the smooth muscle

contractility in lymphatic vessels (80, 106). Through it's combined actions in the spleen and lymphatic system, ANF is able to achieve a sustained decrease in intravascular volume. Under physiological conditions, this would correct the initial perturbation that stimulated ANF release, namely hypervolemia. The complementary actions of ANF in the spleen and the kidney reaffirm the established role of ANF as being responsible for controlling fluid volume homeostasis within the body.

CHAPTER VI

REFERENCES

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REFERENCES

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FIGURES

FIGURES

Figure 1: Schematic diagram showing the structure of the sinusoidal rat spleen. (Reproduced from Groom and Schmidt (1990) in *The spleen: Structure, function and clinical significance*, Chapman and Hall Medical, London.)

Figure 2: Cellular actions of Atrial Natriuretic Factor. ANF binds to natriuretic peptide receptor-A (NPR_A) and stimulates the guanylyl cyclase activity of the receptor. Cyclic guanosine monophosphate (cGMP) exerts its biologic effects indirectly through cGMP-dependent protein kinase G (PKG) or one or more phosphodiesterases (PDEs). ANF also binds to natriuretic peptide receptor-C (NPR_C), which acts as a clearance receptor, and has recently been found to be involved in the anti-mitogenic properties of ANF. Finally, ANF peptide may be degraded by the extracellular neutral endopeptidases (NEPs) in the kidney and vasculature (Reproduced from Levin *et al.* (1998) *New England Journal of Medicine*, **339:** 321-28.)

Figure 3: Anatomy of abdominal blood vessels associated with the spleen: The diagram shows the surgical cannulation sites associated with the isolated blood-perfused spleen preparation. *NB.* The stomach has been reflected from the peritoneal cavity and placed on the thoracic cavity to allow access to the gastric vasculature.

Figure 4: Double vascular occlusion technique for determining capillary pressure. Pressure-time tracings are shown during the double occlusion, where arterial pressure (P_A) and venous pressure (P_V) are shown from the isolated, blood-perfused spleen in A) Control and B) ANF-infused (180 ng min^{-1}) animals.

Figure 5: Effect of ANF on Capillary Pressure (P_C): Hemodynamic alterations in capillary pressure were measure in the blood-perfused spleen (closed-circle) and hindquarters (open-circle) in response to ANF infusion using the double occlusion technique. The vertical error bars delineate SE of the means. $*P < 0.05$.

Figure 6: Effect of ANF on Pre-capillary Resistance (R_A): Hemodynamic alterations in pre-capillary resistance were measure in the blood-perfused spleen (closed-circle) and hindquarters (open-circle) in response to ANF infusion using the double occlusion technique. The vertical error bars delineate SE of the means. $*P < 0.05$.

Figure 7: Effect of ANF on Post-capillary Resistance (R_V): Hemodynamic alterations in post-capillary resistance were measure in the blood-perfused spleen (closed-circle) and hindquarters (open-circle) in response to ANF infusion using the double occlusion technique. The vertical error bars delineate SE of the means. $*P < 0.05$.

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Figure 9: ANF-mediated Changes in Splenic Hemodynamics: ANF dose-dependently increased splenic post-capillary resistance and capillary pressure (**A**). The alterations in post-capillary resistance are directly correlated with changes in capillary pressure (**B**). The vertical error bars delineate SE of the means. $^*P < 0.05$.

Figure 10: Effects of C-ANF on Splenic Hemodynamics: C-ANF (black vertical bars), a NPR_C-specific agonist had no effect on intrasplenic capillary pressure (A) or post-capillary resistance (B) in comparison to ANF (white vertical bars). The vertical error bars delineate SE of the means. $^*P < 0.05$.

Figure 11: Intrinsic Effects of A71915 on Splenic Hemodynamics: A71915 (black vertical bars), an ANF antagonist, had no effect on intrasplenic capillary pressure (A) or post-capillary resistance (B) in comparison to ANF (white vertical bars). The vertical error bars delineate SE of the means. $^*P < 0.05$.

Figure 12: Effects of A71915 on ANF-induced Changes in Splenic Hemodynamics: High doses of A71915 (gray vertical bars) complete abolished

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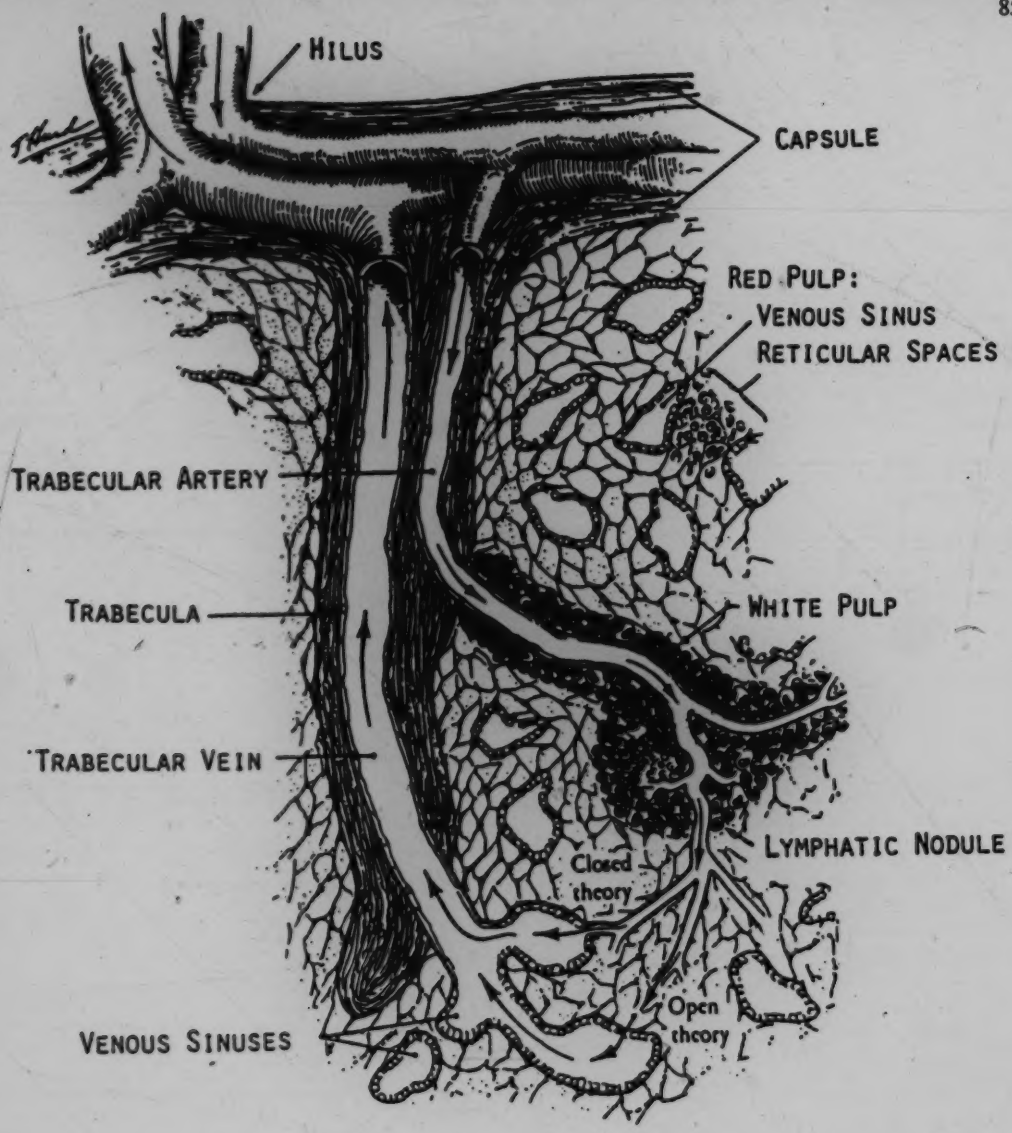

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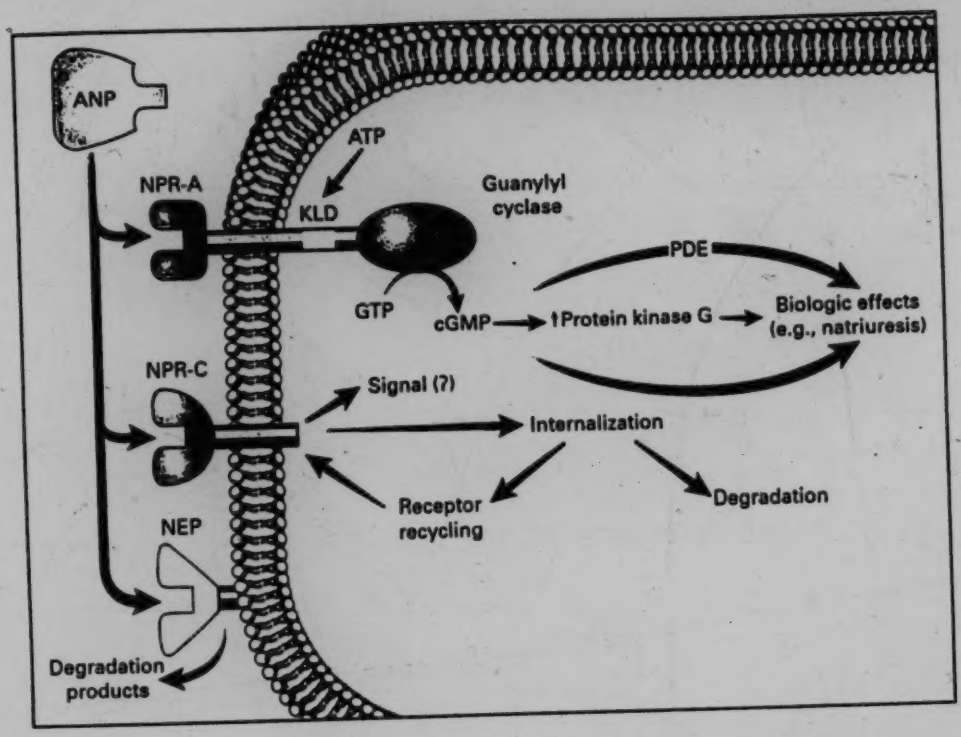

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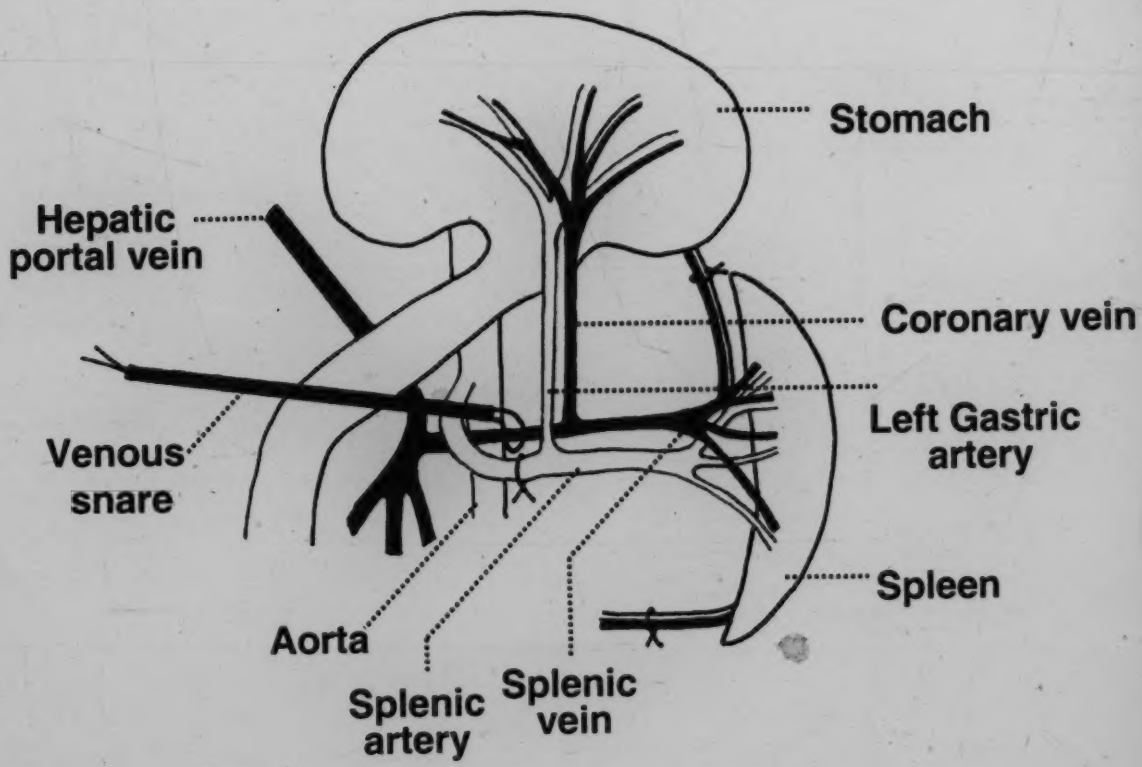

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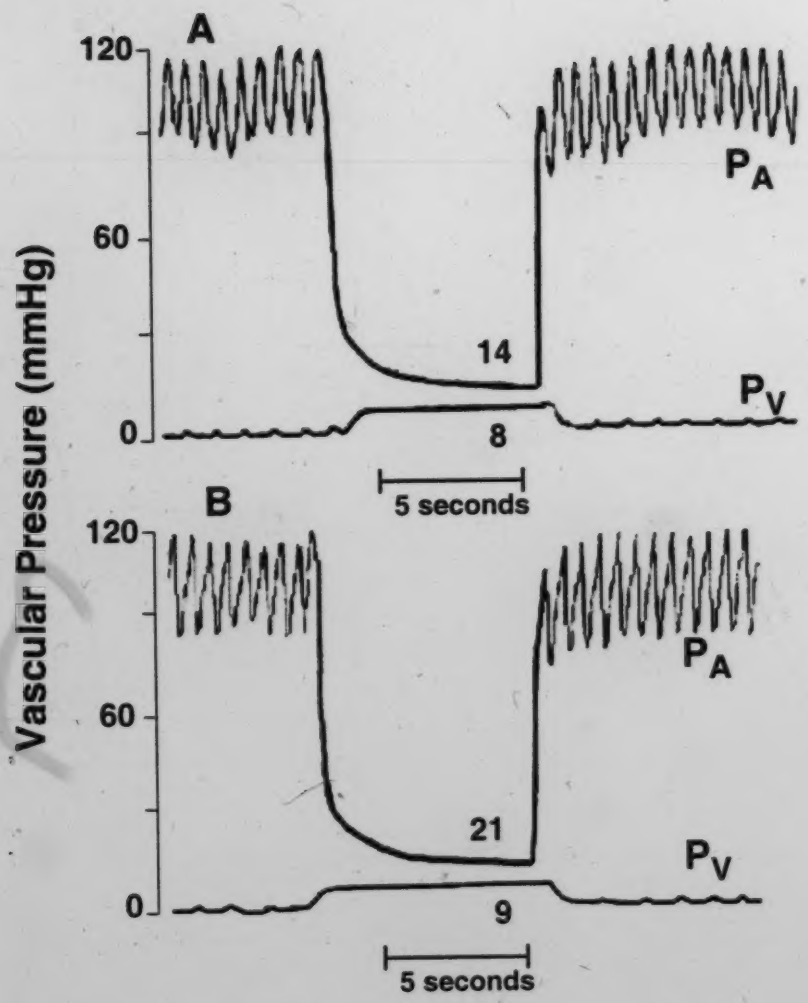

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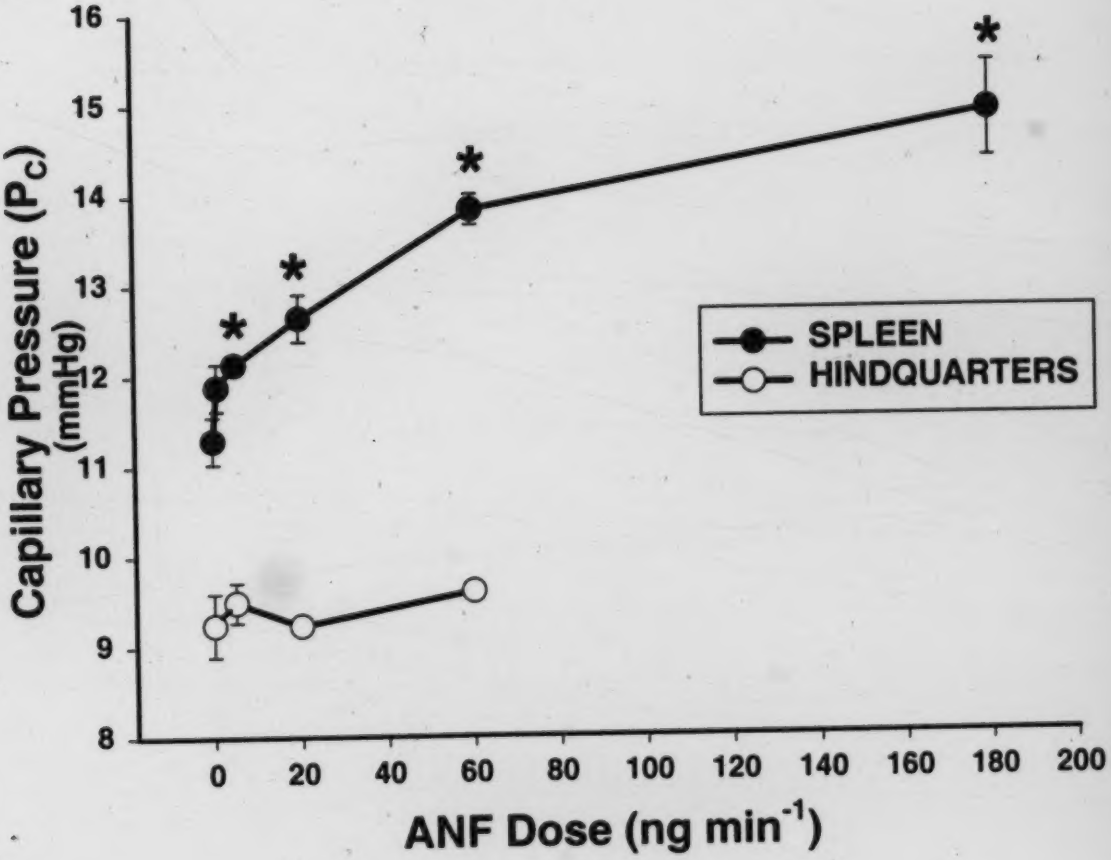

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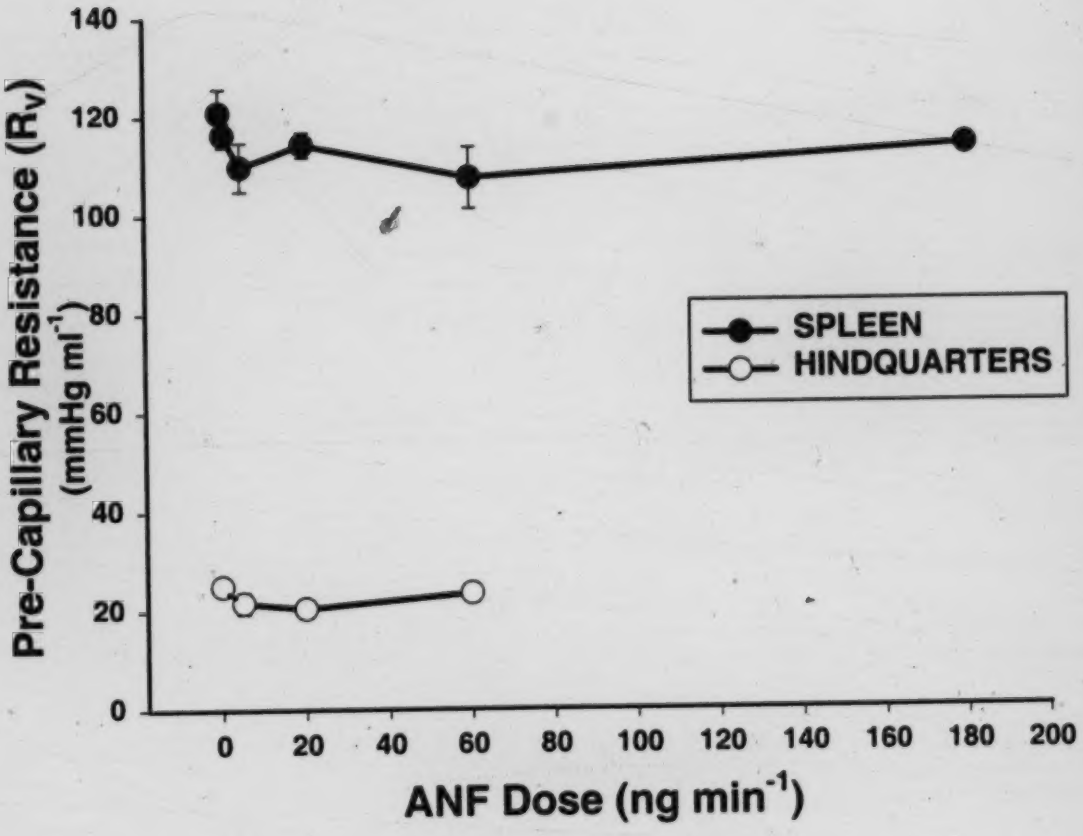

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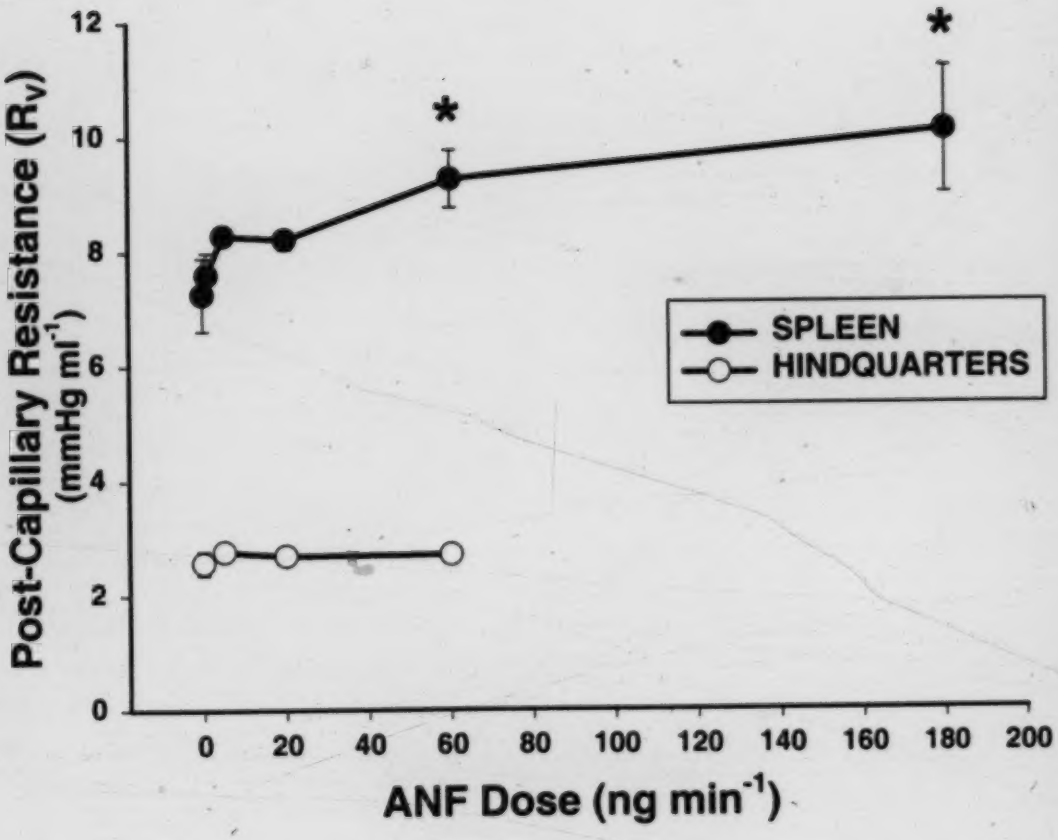

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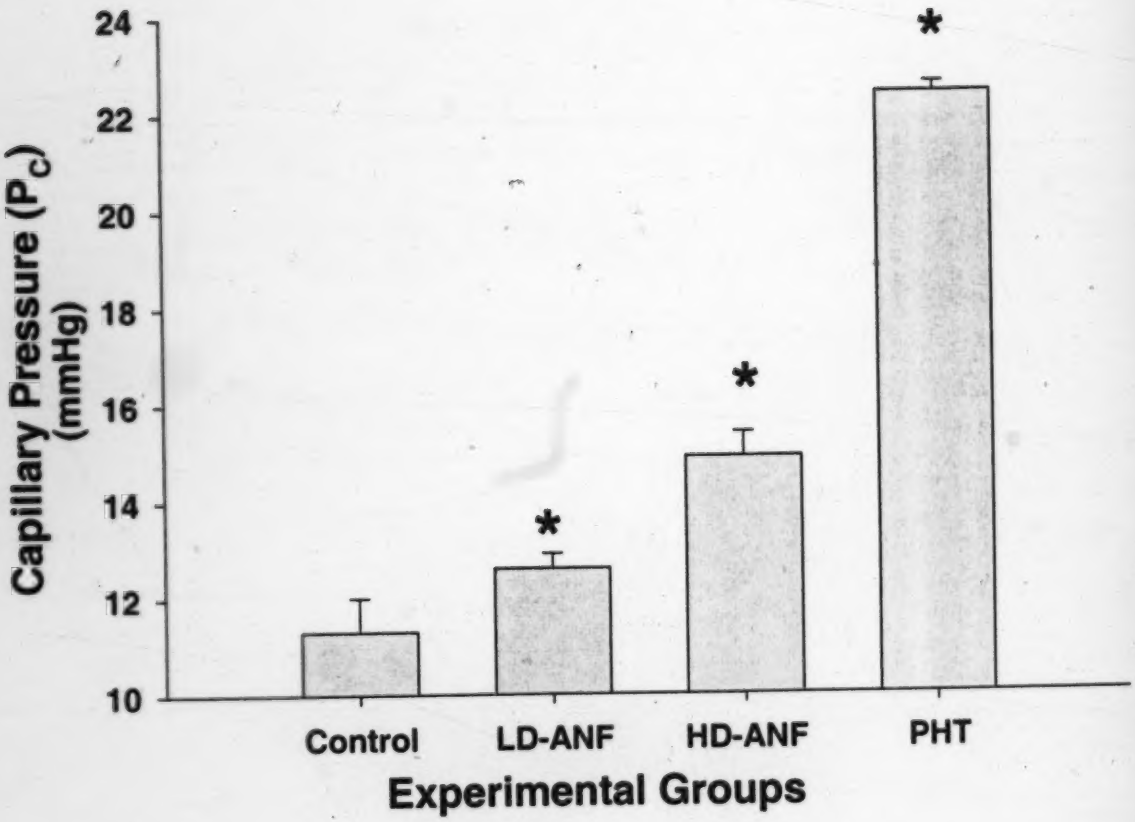

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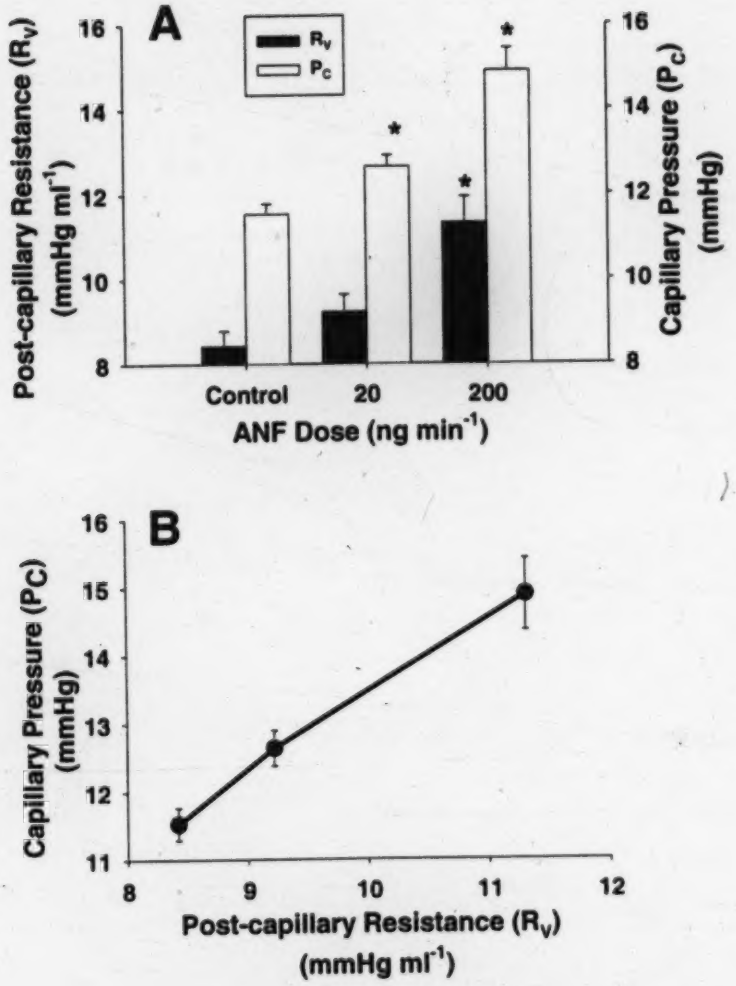

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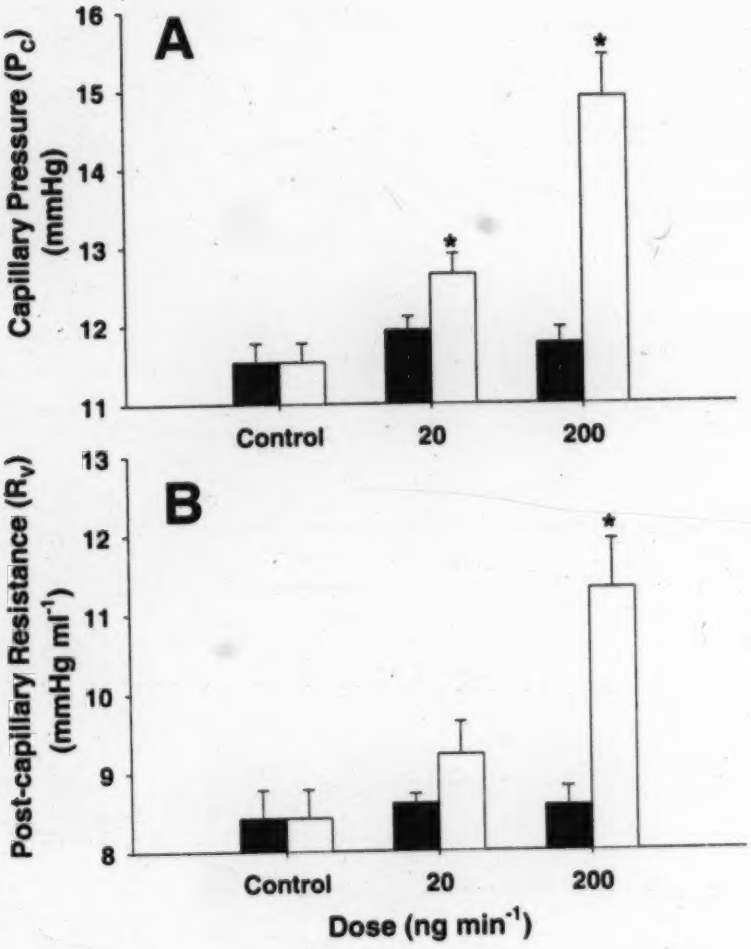

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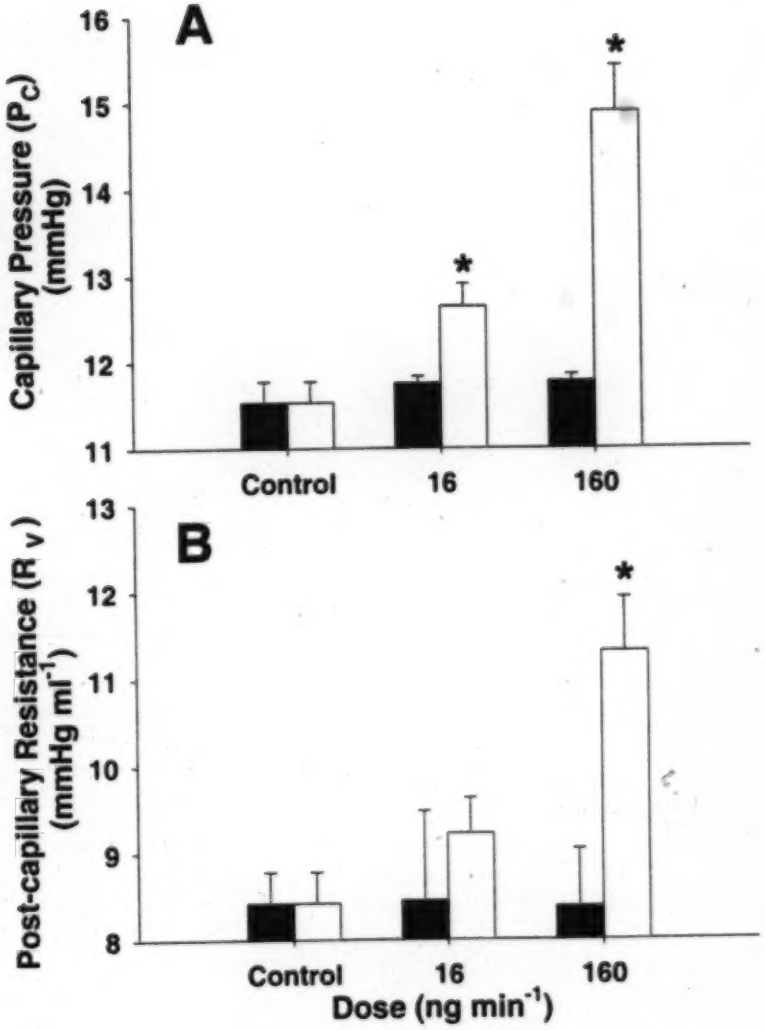

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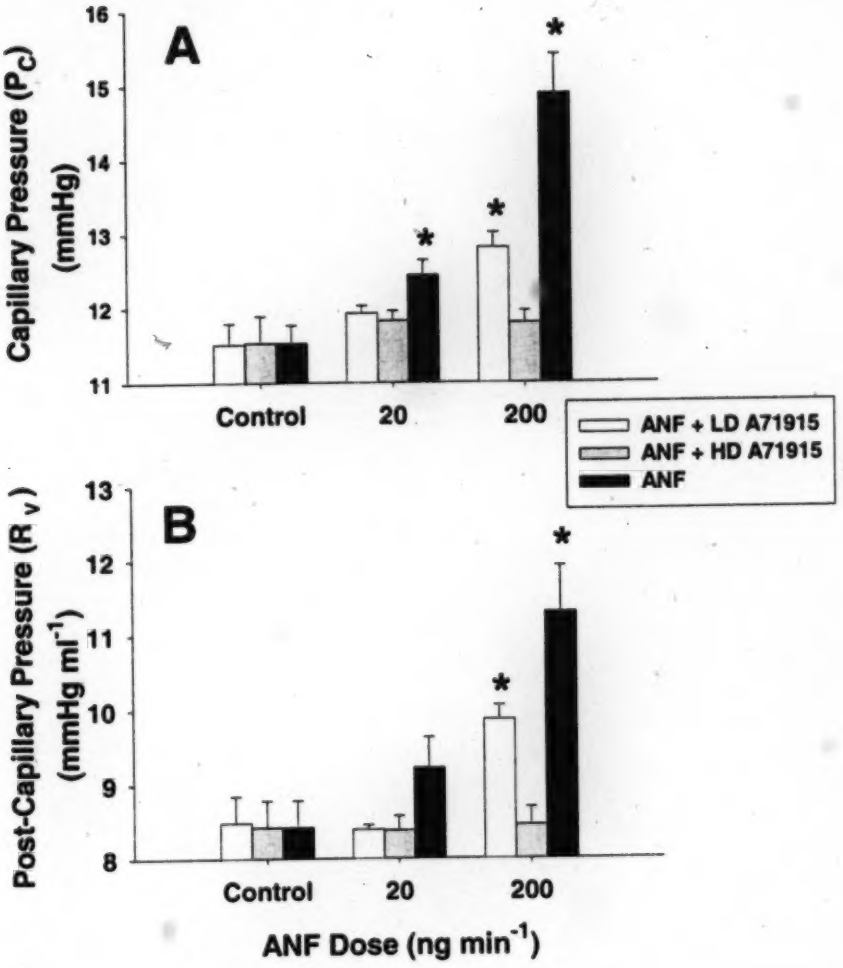

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